(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 November 2005 (10.11.2005)

PCT

(10) International Publication Number
WO 2005/105155 A1

(51) International Patent Classification[7]: A61K 48/00

(21) International Application Number:
PCT/JP2005/008401

(22) International Filing Date: 26 April 2005 (26.04.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/566,138 28 April 2004 (28.04.2004) US

(71) Applicant *(for all designated States except US)*: **SAITAMA MEDICAL SCHOOL** [JP/JP]; 38, Morohongo, Moroyama-machi, Iruma-gun, Saitama, 3500495 (JP).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **MORI, Keisuke** [JP/JP]; 844-1, Tottori-machi, Maebashi-shi, Gunma, 3710131 (JP).

(74) Agent: **TAKASHIMA, Hajime**; Meiji Yasuda Seimei Osaka, Midosuji Bldg., 1-1, Fushimimachi 4-chome, Chuo-ku, Osaka-shi, Osaka, 5410044 (JP).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS FOR DELIVERING A GENE PRODUCT TO THE EYE

(57) Abstract: The invention is directed to a method of delivering a gene product to an eye. The method comprises (a) inducing a stress response in the eye, and (b) subsequently administering to the eye an expression vector comprising a nucleic acid sequence encoding a gene product. The expression vector transduces a host cell and the nucleic acid sequence is expressed to produce the gene product. The invention further is directed to a method of prophylactically or therapeutically treating an animal for an ocular-related disorder. The method comprises (a) inducing a stress response in an eye, and (b) subsequently administering to the eye an expression vector comprising a nucleic acid sequence encoding an inhibitor of angiogenesis and/or a neurotrophic agent such that the expression vector transduces a host cell and the nucleic acid sequence is expressed to produce the inhibitor of angiogenesis and/or neurotrophic agent to treat prophylactically or therapeutically the ocular-related disorder.

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DESCRIPTION

METHODS FOR DELIVERING A GENE PRODUCT TO THE EYE

TECHNICAL FIELD

The invention relates to a method of delivering a gene product to the eye.

BACKGROUND ART

An overwhelming majority of the world's population will experience some degree of vision loss in their lifetime. Vision loss affects virtually all people regardless of age, race, economic or social status, or geographical location. Ocular-related disorders, while often not life threatening, necessitate life-style changes that jeopardize the independence of the afflicted individual. Vision impairment can result from most all ocular disorders, including diabetic retinopathies, proliferative retinopathies, retinal detachment, toxic retinopathies, retinal vascular diseases, retinal degenerations, vascular anomalies, age-related macular degeneration and other acquired disorders, infectious diseases, inflammatory diseases, ocular ischemia, pregnancy-related disorders, retinal tumors, choroidal tumors, choroidal disorders, vitreous disorders, trauma, cataract complications, dry eye, and inflammatory optic neuropathies.

Leading causes of severe vision loss and blindness are ocular-related disorders wherein the vasculature of the eye is damaged or insufficiently regulated. Ocular-related diseases comprising a neovascularization aspect are many and include, for example, exudative age-related macular degeneration, diabetic retinopathy, corneal neovascularization, choroidal neovascularization, neovascular glaucoma, cyclitis, Hippel-Lindau Disease, retinopathy of prematurity, pterygium, histoplasmosis, iris neovascularization, macular edema, glaucoma-associated neovascularization, drug-related toxicities, central and branched retinal vein occlusions, and the like. Damage of the retina, i.e., retinal detachment, retinal tears, or retinal degeneration, is directly connected to vision loss. While a common cause of retinal detachment, retinal tears, and retinal degeneration is abnormal, i.e., uncontrolled, vascularization of various ocular tissues, this is not always the case. Atrophic complications associated with age-related macular degeneration, nonproliferative diabetic retinopathy, and inflammatory ocular damage are not associated with neovascularization, but can result in severe vision loss if not treated. Disorders associated with both neovascular and atrophic components, such as age-related macular degeneration and diabetic retinopathy, are particularly difficult to treat due to the emergence of a wide variety of complications.

For many ocular-related disorders, no efficient therapeutic options currently are available. Laser photocoagulation involves administering laser burns to various areas of the eye and is used in the treatment of many neovascularization-linked disorders. For example, focal macular photocoagulation is used to treat areas of vascular leakage outside the macula

(Murphy, *Amer. Family Physician, 51*(4), 785-796 (1995)). Similarly, neovascularization, in particular, advanced proliferative retinopathy, is commonly treated with scatter or panretinal photocoagulation. Laser treatment does not guarantee that vision loss will be attenuated. In fact, many patients afflicted with age-related macular degeneration eventually experience severe vision loss in spite of treatment. Other treatment options for ocular-related disorders include thermotherapy, radiation therapy, surgery, e.g., macular translocation, removal of excess ocular tissue, drug therapy, and the like. However, in most cases, all available treatment options have limited therapeutic effect, require repeated, costly procedures, and/or are associated with dangerous side-effects.

Given the prevalence of ocular-related disorders, there remains a need for an effective prophylactic and therapeutic treatment of ocular-related disorders. Accordingly, the invention provides materials and methods for delivering a gene product, such as a therapeutic gene product, to the eye. This and other advantages of the invention will become apparent from the detailed description provided herein.

DISCLOSURE OF INVENTION

The invention is directed to a method of delivering a gene product to an eye. The method comprises (a) inducing a stress response in the eye, and (b) subsequently administering to the eye an expression vector comprising a nucleic acid sequence encoding a gene product, wherein the expression vector transduces a host cell and the nucleic acid sequence is expressed to produce the gene product. The invention further provides a method of prophylactically or therapeutically treating an animal for an ocular-related disorder. The method comprises (a) inducing a stress response in an eye, and (b) subsequently administering to the eye an expression vector comprising a nucleic acid sequence encoding an inhibitor of angiogenesis and/or a neurotrophic agent such that the expression vector transduces a host cell and the nucleic acid sequence is expressed to produce the inhibitor of angiogenesis and/or neurotrophic agent to treat prophylactically or therapeutically the ocular-related disorder. Preferably, inducing a stress response in the eye comprises applying photodynamic therapy or photocoagulation therapy to the eye. Also preferably, the expression vector is an adenoviral vector.

BEST MODE FOR CARRYING OUT THE INVENTION

The invention is directed to a method of delivering a gene product, such as a therapeutic gene product, to the eye. The method comprises (a) inducing a stress response in the eye, and (b) subsequently administering to the eye an expression vector comprising a nucleic acid sequence encoding a gene product, wherein the expression vector transduces a host cell and the nucleic acid sequence is expressed to produce the gene product. Any gene product can be delivered to the eye in accordance with the inventive method, including therapeutic gene products, diagnostic gene products, gene products used for research

purposes (e.g., marker proteins or antigenic proteins), and the like. However, the gene product delivered to the eye preferably is a therapeutic gene product for the prophylactic or therapeutic treatment of an animal, preferably a human, for at least one ocular-related disorder. Ocular-related disorders appropriate for treatment using the inventive method include, but are not limited to, diabetic retinopathies, proliferative retinopathies, retinopathy of prematurity, retinal vascular diseases, vascular anomalies, age-related macular degeneration and other acquired disorders, endophthalmitis, infectious diseases, inflammatory diseases, AIDS-related disorders, ocular ischemia syndrome, pregnancy-related disorders, peripheral retinal degenerations, retinal degenerations, toxic retinopathies, cataracts, retinal tumors, corneal neovascularization, choroidal tumors, choroidal disorders, choroidal neovascularization, neovascular glaucoma, vitreous disorders, retinal detachment and proliferative vitreoretinopathy, cyclitis, non-penetrating trauma, penetrating trauma, post-cataract complications, Hippel-Lindau Disease, dry eye, inflammatory optic neuropathies, glaucoma, macular edema, pterygium, iris neovascularization, uveitis, pathologic myopia, surgical-induced disorders, and the like.

The invention further provides a method of prophylactically or therapeutically treating an animal for at least one ocular-related disorder, such as ocular neovascularization. The method comprises (a) inducing a stress response in an eye, and (b) subsequently administering to the eye an expression vector comprising a nucleic acid sequence encoding an inhibitor of angiogenesis and/or a neurotrophic agent such that the expression vector transduces a host cell and the nucleic acid sequence is expressed to produce the inhibitor of angiogenesis and/or neurotrophic agent to treat prophylactically or therapeutically the ocular-related disorder. Preferably, the expression vector comprises a nucleic acid sequence encoding an inhibitor of angiogenesis and the same or different nucleic acid sequence encoding a neurotrophic agent. Desirably, the nucleic acid sequence encodes pigment epithelium-derived factor (PEDF).

The ocular disorder preferably is ocular neovascularization, such as neovascularization of the choroid. The choroid is a thin, vascular membrane located under the retina. Abnormal neovascularization of the choroid results from, for example, photocoagulation, anterior ischemic optic neuropathy, Best's disease, choroidal hemangioma, metallic intraocular foreign body, choroidal nonperfusion, choroidal osteomas, choroidal rupture, bacterial endocarditis, choroideremia, chronic retinal detachment, drusen, deposit of metabolic waste material, endogenous *Candida* endophthalmitis, neovascularization at ora serrata, operating microscope burn, punctate inner choroidopathy, radiation retinopathy, retinal cryoinjury, retinitis pigmentosa, retinochoroidal coloboma, rubella, subretinal fluid drainage, tilted disc syndrome, *Taxoplasma* retinochoroiditis, tuberculosis, and the like.

Neovascularization of the cornea also is appropriate for treatment by the method of the invention. The cornea is a projecting, transparent section of the fibrous tunic, the outer most layer of the eye. The outermost layer of the cornea contacts the conjunctiva, while the

innermost layer comprises the endothelium of the anterior chamber. Corneal neovascularization stems from, for example, ocular injury, surgery, infection, improper wearing of contact lenses, and diseases such as, for example, corneal dystrophies.

Alternatively, the ocular neovascularization is neovascularization of the retina. Retinal neovascularization is an indication associated with numerous ocular diseases and disorders, many of which are named above. Preferably, the neovascularization of the retina treated in accordance with the inventive method is associated with diabetic retinopathy. Common causes of retinal neovascularization include ischemia, viral infection, and retinal damage. Neovascularization of the retina can lead to macular edema, subretinal discoloration, scarring, hemorrhaging, and the like. Complications associated with retina neovascularization stem from growth, breakage, and leakage of newly formed blood vessels. Vision is impaired as blood fills the vitreous cavity and is not efficiently removed. Not only is the passage of light impeded, but an inflammatory response to the excess blood and metabolites can cause further damage to ocular tissue. In addition, the new vessels form fibrous scar tissue, which, over time, will disturb the retina causing retinal tears and detachment.

The ocular disorder can be age-related macular degeneration, which can involve both exudative (neovascular) and atrophic complications. Exudative complications include, for example, disciform scars (i.e., scarring involving fibrous elements) and neovascularization. Atrophic complications include, for instance, the formation of drusen and basal laminar deposits, irregularity of retinal pigmentation, and accumulation of lipofuscin granules. The ocular disorder also can be ocular edema (e.g., retinal edema or macular edema).

By "prophylactic" is meant the protection, in whole or in part, against ocular-related disorders, in particular ocular neovascularization or age-related macular degeneration. By "therapeutic" is meant the amelioration of the ocular-related disorder, itself, and the protection, in whole or in part, against further ocular-related disease, in particular ocular neovascularization or age-related macular degeneration. One of ordinary skill in the art will appreciate that any degree of protection from, or amelioration of, an ocular-related disorder is beneficial to a patient.

The inventive method can be used to treat both acute and persistent, progressive ocular-related disorders. For acute ailments, the expression vector can be administered using a single or multiple applications within a short time period. For persistent ocular-related diseases, such as age-related macular degeneration and diabetic retinopathy, numerous applications of the expression vector may be necessary to realize a therapeutic effect.

The inventive method comprises inducing a stress response in the eye prior to administering an expression vector. It has surprisingly been determined that treating an eye with, for example, photocoagulation or photodynamic therapy increases in ocular cells the expression of cell surface molecules which facilitate transduction by expression vectors and cell adhesion. In particular, the cell surface molecules most recognized as mediating

adenoviral infection, coxsackievirus and adenovirus receptor (CAR) and integrins (e.g., integrins β3 and β5), are upregulated in the retina and choroid following, for example, photocoagulation therapy. Upregulation of CAR and integrins allows transduction of a greater number of host cells with an adenoviral vector encoding a gene product as compared to adenoviral transduction efficiency without induction of the stress response (e.g., laser therapy). Accordingly, the level of transduction of host cells by the expression vector is enhanced as compared to the level of transduction of host cells by the expression vector in the absence of inducing a stress response in the eye. In some instances, the invention allows delivery of a smaller dose of expression vector than previously thought possible to achieve a desired biological response.

In addition to increasing transduction of host cells by the expression vector, inducing a stress response in the eye prior to administering the expression vector enhances and prolongs expression of the nucleic acid sequence of the expression vector compared to expression of the nucleic acid sequence in the absence of the stress response. Ideally, expression of the nucleic acid sequence in the context of the inventive method is enhanced compared to expression of the nucleic acid sequence in the absence of inducing the stress response in the eye (but under otherwise similar conditions) for at least one day (preferably at least 3 days (e.g., 1, 2, or 3 days) or at least five days) following administration of the expression vector. More preferably, expression of the nucleic acid sequence is enhanced for at least 7 days (e.g., at least 14 days or at least 21 days) post-administration of the expression vector as compared to expression of the nucleic acid sequence in the absence of the stress response (e.g., in the absence of photocoagulation therapy) at the same timepoint. Even more preferably, expression of the nucleic acid sequence is enhanced as compared to expression of the nucleic acid sequence in the absence of inducing a stress response for at least 28 days (e.g., at least 60 days or at least 90 days) post-administration of the expression vector to the eye.

By "enhanced" expression is meant any increase in transcription compared to transcription (i.e., gene or nucleic acid sequence expression) which occurs in the absence of inducing the stress response. Any increase in expression of the nucleic acid sequence is appropriate in the context of the invention. For example, enhanced expression of the nucleic acid sequence can be a 2-, 3-, 5-, 10-, 20-, or 50-fold increase in expression as compared to the level of gene expression which occurs under similar conditions but in the absence of inducing the stress response in the eye. The enhanced expression (transcription) can result in increased levels of RNA transcript, increased protein production, and/or an enhancement in detectable gene product activity, all of which can be detected using routine laboratory techniques.

A stress response can be induced in the eye by exposure to heat using, for example, lasers in photodynamic therapy, exposure to cold, exposure to light, exposure to radiation (e.g., X-rays), exposure to microwaves, exposure to ultrasound, or physical trauma, all of which can alter the ocular cellular environment to enhance transcription. Desirably, inducing

a stress response in the eye comprises applying photodynamic therapy or photocoagulation therapy to the eye.

One of ordinary skill in the art will appreciate that any of a number of expression vectors known in the art are suitable for use in the inventive method. Examples of suitable expression vectors include, for instance, plasmids, plasmid-liposome complexes, and viral vectors, e.g., parvoviral-based vectors (i.e., adeno-associated virus (AAV)-based vectors), retroviral vectors, herpes simplex virus (HSV)-based vectors, AAV-adenoviral chimeric vectors, and adenovirus-based vectors. Any of these expression vectors can be prepared using standard recombinant DNA techniques described in, e.g., Sambrook et al., *Molecular Cloning, a Laboratory Manual*, 2d edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989), and Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley & Sons, New York, N.Y. (1994).

Plasmids, genetically engineered circular double-stranded DNA molecules, can be designed to contain an expression cassette for delivery of the nucleic acid sequence encoding the gene product to a host cell, such as an ocular cell. Although plasmids were the first vector described for administration of therapeutic nucleic acids, the level of transfection efficiency is poor compared with other techniques. By complexing the plasmid with liposomes, the efficiency of gene transfer in general is improved. While the liposomes used for plasmid-mediated gene transfer strategies have various compositions, they are typically synthetic cationic lipids. Advantages of plasmid-liposome complexes include their ability to transfer large pieces of DNA encoding a therapeutic nucleic acid and their relatively low immunogenicity.

Plasmids are often used for short-term expression. However, a plasmid construct can be modified to obtain prolonged expression. It has recently been discovered that the inverted terminal repeats (ITR) of parvovirus, in particular adeno-associated virus (AAV), are responsible for the high-level persistent nucleic acid expression often associated with AAV (see, for example, U.S. Patent 6,165,754). Accordingly, the expression vector can be a plasmid comprising native parvovirus ITRs to obtain prolonged and substantial expression of a nucleic acid sequence encoding a gene product, e.g., at least one inhibitor of angiogenesis and/or at least one neurotrophic factor. While plasmids are suitable for use in the inventive method, preferably the expression vector is a viral vector.

AAV vectors are viral vectors of particular interest for use in gene therapy protocols. AAV is a DNA virus, which is not known to cause human disease. AAV requires co-infection with a helper virus (i.e., an adenovirus or a herpes virus), or expression of helper genes, for efficient replication. AAV vectors used for administration of a therapeutic nucleic acid have approximately 96% of the parental genome deleted, such that only the terminal repeats (ITRs), which contain recognition signals for DNA replication and packaging, remain. This eliminates immunologic or toxic side effects due to expression of viral genes. In addition, delivering the AAV *rep* protein enables integration of the AAV vector comprising

AAV ITRs into a specific region of genome, if desired. Host cells comprising an integrated AAV genome show no change in cell growth or morphology (see, for example, U.S. Patent 4,797,368).

Retrovirus is an RNA virus capable of infecting a wide variety of host cells. Upon infection, the retroviral genome integrates into the genome of its host cell and is replicated along with host cell DNA, thereby constantly producing viral RNA and any nucleic acid sequence incorporated into the retroviral genome. When employing pathogenic retroviruses, e.g., human immunodeficiency virus (HIV) or human T-cell lymphotrophic viruses (HTLV), care must be taken in altering the viral genome to eliminate toxicity. A retroviral vector can additionally be manipulated to render the virus replication-incompetent. As such, retroviral vectors are thought to be particularly useful for stable gene transfer *in vivo*. Lentiviral vectors, such as HIV-based vectors, are exemplary of retroviral vectors used for gene delivery. Unlike other retroviruses, HIV-based vectors are known to incorporate their passenger genes into non-dividing cells and, therefore, can be of use in treating atrophic forms of ocular-related disease.

HSV-based viral vectors are suitable for use as an expression vector to introduce nucleic acids into host cells (e.g., ocular cells). The mature HSV virion consists of an enveloped icosahedral capsid with a viral genome consisting of a linear double-stranded DNA molecule that is 152 kb. Most replication-deficient HSV vectors contain a deletion to remove one or more intermediate-early genes to prevent replication. Advantages of the herpes vector are its ability to enter a latent stage that can result in long-term DNA expression, and its large viral DNA genome that can accommodate exogenous DNA up to 25 kb. Of course, this ability is also a disadvantage in terms of short-term treatment regimens. For a description of HSV-based vectors appropriate for use in the inventive method, see, for example, U.S. Patents 5,837,532; 5,846,782; 5,849,572; and 5,804,413; and International Patent Applications WO 91/02788, WO 96/04394, WO 98/15637, and WO 99/06583.

Adenovirus (Ad) is a 36 kb double-stranded DNA virus that efficiently transfers DNA *in vivo* to a variety of different target cell types. For use in the inventive method, the virus is preferably made replication deficient by deleting select genes required for viral replication. The expendable E3 region is also frequently deleted to allow additional room for a larger DNA insert. The vector can be produced in high titers and can efficiently transfer DNA to replicating and non-replicating cells. The newly transferred genetic information remains epi-chromosomal, thus eliminating the risks of random insertional mutagenesis and permanent alteration of the genotype of the target cell. However, if desired, the integrative properties of AAV can be conferred to adenovirus by constructing an AAV-Ad chimeric vector. For example, the AAV ITRs and nucleic acid encoding the Rep protein incorporated into an adenoviral vector enables the adenoviral vector to integrate into a mammalian cell genome. Therefore, AAV-Ad chimeric vectors are an interesting option for use in the invention.

Preferably, the expression vector of the inventive method is a viral vector; more preferably, the expression vector is an adenoviral vector, e.g., a human adenoviral vector. In the context of the invention, the adenoviral vector can be derived from any serotype of adenovirus. Adenoviral stocks that can be employed as a source of adenovirus can be amplified from the adenoviral serotypes 1 through 51, which are currently available from the American Type Culture Collection (ATCC, Manassas, VA), or from any other serotype of adenovirus available from any other source. For instance, an adenovirus can be of subgroup A (e.g., serotypes 12, 18, and 31), subgroup B (e.g., serotypes 3, 7, 11, 14, 16, 21, 34, and 35), subgroup C (e.g., serotypes 1, 2, 5, and 6), subgroup D (e.g., serotypes 8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, and 42-47), subgroup E (serotype 4), subgroup F (serotypes 40 and 41), or any other adenoviral serotype. Preferably, however, an adenovirus is of serotype 2, 5, or 9. However, non-group C adenoviruses can be used to prepare replication-deficient adenoviral gene transfer vectors for delivery of gene products to host cells, such as ocular cells. Preferred adenoviruses used in the construction of non-group C adenoviral gene transfer vectors include Ad12 (group A), Ad7 and Ad35 (group B), Ad30 and Ad36 (group D), Ad4 (group E), and Ad41 (group F). Non-group C adenoviral vectors, methods of producing non-group C adenoviral vectors, and methods of using non-group C adenoviral vectors are disclosed in, for example, U.S. Patents 5,801,030; 5,837,511; and 5,849,561 and International Patent Applications WO 97/12986 and WO 98/53087.

The adenoviral vector is preferably deficient in at least one gene function required for viral replication, thereby resulting in a "replication-deficient" adenoviral vector. By "replication-deficient" is meant that the adenoviral vector comprises an adenoviral genome that lacks at least one replication-essential gene function (i.e., such that the adenoviral vector does not replicate in typical host cells, especially those in the human patient that could be infected by the adenoviral vector in the course of treatment in accordance with the invention). A deficiency in a gene, gene function, or gene or genomic region, as used herein, is defined as a deletion of sufficient genetic material of the viral genome to impair or obliterate the function of the gene whose nucleic acid sequence was deleted in whole or in part. Deletion of an entire gene region often is not required for disruption of a replication-essential gene function. However, for the purpose of providing sufficient space in the adenoviral genome for one or more transgenes, removal of a majority of a gene region may be desirable. Replication-essential gene functions are those gene functions that are required for replication (e.g., propagation) and are encoded by, for example, the adenoviral early regions (e.g., the E1, E2, and E4 regions), late regions (e.g., the L1-L5 regions), genes involved in viral packaging (e.g., the IVa2 gene), and virus-associated RNAs (e.g., VA-RNA-1 and/or VA-RNA-2). More preferably, the replication-deficient adenoviral vector comprises an adenoviral genome deficient in at least one replication-essential gene function of one or more regions of the adenoviral genome. In this respect, the adenoviral vector is deficient in at least one essential gene function of the E1 region of the adenoviral genome required for viral replication. In

addition to a deficiency in the E1 region, the recombinant adenovirus can also have a mutation in the major late promoter (MLP). The mutation in the MLP can be in any of the MLP control elements such that it alters the responsiveness of the promoter, as discussed in International Patent Application WO 00/00628. More preferably, the vector is deficient in at least one essential gene function of the E1 region and at least part of the E3 region (e.g., an Xba I deletion of the E3 region). With respect to the E1 region, the adenoviral vector can be deficient in at least part of the E1a region and at least part of the E1b region. For example, the adenoviral vector can comprise a deletion of the entire E1 region and part of the E3 region of the adenoviral genome (i.e., nucleotides 355 to 3,511 and 28,593 to 30,470). A singly-deficient adenoviral vector can be deleted of approximately nucleotides 356 to 3,329 and 28,594 to 30,469 (based on the adenovirus serotype 5 genome). Alternatively, the adenoviral vector genome can be deleted of approximately nucleotides 356 to 3,510 and 28,593 to 30,470 (based on the adenovirus serotype 5 genome). The endpoints defining the deleted nucleotide portions can be difficult to precisely determine and typically will not significantly affect the nature of the adenoviral vector, i.e., each of the aforementioned nucleotide numbers can be +/- 1, 2, 3, 4, 5, or even 10 or 20 nucleotides.

Preferably, the adenoviral vector is "multiply deficient," meaning that the adenoviral vector is deficient in one or more essential gene functions required for viral replication in each of two or more regions. For example, the aforementioned E1-deficient or E1-, E3-deficient adenoviral vectors can be further deficient in at least one essential gene function of the E4 region. Adenoviral vectors deleted of the entire E4 region can elicit lower host immune responses. When E4-deficient, the adenoviral vector genome can comprise a deletion of, for example, nucleotides 32,826 to 35,561 (based on the adenovirus serotype 5 genome), optionally in addition to deletions in the E1 region (e.g., nucleotides 356 to 3,329 or nucleotides 356 to 3,510) and/or deletions in the E3 region (e.g., nucleotides 28,594 to 30,469 or nucleotides 28,593 to 30,470).

Alternatively, the adenoviral vector lacks all or part of the E1 region and all or part of the E2 region (e.g., the E2A region). However, adenoviral vectors lacking all or part of the E1 region, all or part of the E2 region, and all or part of the E3 region also are contemplated herein. In one embodiment, the adenoviral vector lacks all or part of the E1 region, all or part of the E2 region, all or part of the E3 region, and all or part of the E4 region. Suitable replication-deficient adenoviral vectors are disclosed in U.S. Patents 5,851,806 and 5,994,106 and International Patent Applications WO 95/34671 and WO 97/21826. For example, suitable replication-deficient adenoviral vectors include those with at least a partial deletion of the E1a region, at least a partial deletion of the E1b region, at least a partial deletion of the E2a region, and at least a partial deletion of the E3 region. Alternatively, the replication-deficient adenoviral vector can have at least a partial deletion of the E1 region, at least a partial deletion of the E3 region, and at least a partial deletion of the E4 region. Alternatively or in addition, other regions of the adenoviral genome also can be deleted such

as the VAI gene and VAII gene as described in International Patent Application No. PCT/US02/29111. Multiply-deficient viral vectors are particularly useful in that such vectors can accept large inserts of exogenous DNA. Indeed, adenoviral amplicons, an example of a multiply-deficient adenoviral vector which comprises only those genomic sequences required for packaging and replication of the viral genome, can accept inserts of approximately 36 kb.

Therefore, in a preferred embodiment, the expression vector of the inventive method is a multiply-deficient adenoviral vector lacking all or part of the E1 region, all or part of the E3 region, all or part of the E4 region, and, optionally, all or part of the E2 region. In this regard, it has been observed that an at least E4-deficient adenoviral vector expresses a transgene at high levels for a limited amount of time *in vivo* and that persistence of expression of a transgene in an at least E4-deficient adenoviral vector can be modulated through the action of a trans-acting factor, such as HSV ICP0, Ad pTP, CMV-IE2, CMV-IE86, HIV tat, HTLV-tax, HBV-X, AAV Rep 78, the cellular factor from the U205 osteosarcoma cell line that functions like HSV ICP0, or the cellular factor in PC12 cells that is induced by nerve growth factor, among others. In view of the above, a nucleic acid sequence encoding a trans-acting factor that modulates the persistence of expression of the nucleic acid sequence encoding the gene product can be administered. Use of trans-acting factors in combination with replication deficient adenoviral vectors is further described in U.S. Patents 6,225,113; 6,660,521; and 6,649,373; and International Patent Application WO 00/34496.

It should be appreciated that the deletion of different regions of the adenoviral vector can alter the immune response of the mammal. In particular, deletion of different regions can reduce the inflammatory response generated by the adenoviral vector. Furthermore, the adenoviral vector's coat protein can be modified so as to decrease the adenoviral vector's ability or inability to be recognized by a neutralizing antibody directed against the wild-type coat protein, as described in International Patent Application WO 98/40509. Such modifications are useful for long-term treatment of persistent ocular disorders.

The adenoviral vector, when multiply replication-deficient, especially in replication-essential gene functions of the E1 and E4 regions, preferably includes a spacer element to provide viral growth in a complementing cell line similar to that achieved by singly replication-deficient adenoviral vectors, particularly an adenoviral vector comprising a deficiency in the E1 region. In a preferred E4$^-$ adenoviral vector of the invention wherein the L5 fiber region is retained, the spacer is desirably located between the L5 fiber region and the right-side ITR. More preferably in such an adenoviral vector, the E4 polyadenylation sequence alone or, most preferably, in combination with another sequence exists between the L5 fiber region and the right-side ITR, so as to sufficiently separate the retained L5 fiber region from the right-side ITR, such that viral production of such a vector approaches that of a singly replication deficient adenoviral vector, particularly a singly replication deficient E1 deficient adenoviral vector.

The spacer element can contain any sequence or sequences which are of a desired length, such as sequences at least about 15 base pairs (e.g., between about 15 base pairs and about 12,000 base pairs), preferably about 100 base pairs to about 10,000 base pairs, more preferably about 500 base pairs to about 8,000 base pairs, even more preferably about 1,500 base pairs to about 6,000 base pairs, and most preferably about 2,000 to about 3,000 base pairs in length. The spacer element sequence can be coding or non-coding and native or non-native with respect to the adenoviral genome, but does not restore the replication-essential function to the deficient region. The spacer can also contain a promoter-variable expression cassette. More preferably, the spacer comprises an additional polyadenylation sequence and/or a passenger gene. Preferably, in the case of a spacer inserted into a region deficient for E4, both the E4 polyadenylation sequence and the E4 promoter of the adenoviral genome or any other (cellular or viral) promoter remain in the vector. The spacer is located between the E4 polyadenylation site and the E4 promoter, or, if the E4 promoter is not present in the vector, the spacer is proximal to the right-side ITR. The spacer can comprise any suitable polyadenylation sequence. Examples of suitable polyadenylation sequences include synthetic optimized sequences, BGH (Bovine Growth Hormone), polyoma virus, TK (Thymidine Kinase), EBV (Epstein Barr Virus) and the papillomaviruses, including human papillomaviruses and BPV (Bovine Papilloma Virus). Preferably, particularly in the E4 deficient region, the spacer includes an SV40 polyadenylation sequence. The SV40 polyadenylation sequence allows for higher virus production levels of multiply replication deficient adenoviral vectors. In the absence of a spacer, production of fiber protein and/or viral growth of the multiply replication-deficient adenoviral vector is reduced by comparison to that of a singly replication-deficient adenoviral vector. However, inclusion of the spacer in at least one of the deficient adenoviral regions, preferably the E4 region, can counteract this decrease in fiber protein production and viral growth. Ideally, the spacer is composed of the glucuronidase gene. The use of a spacer in an adenoviral vector is further described in, for example, U.S. Patent 5,851,806 and International Patent Application WO 97/21826.

Desirably, the adenoviral vector requires, at most, complementation of replication-essential gene functions of the E1, E2A, and/or E4 regions of the adenoviral genome for replication (i.e., propagation). However, the adenoviral genome can be modified to disrupt one or more replication-essential gene functions as desired by the practitioner, so long as the adenoviral vector remains deficient and can be propagated using, for example, complementing cells and/or exogenous DNA (e.g., helper adenovirus) encoding the disrupted replication-essential gene functions. In this respect, the adenoviral vector can be deficient in replication-essential gene functions of only the early regions of the adenoviral genome, only the late regions of the adenoviral genome, and both the early and late regions of the adenoviral genome. The adenoviral vector also can have essentially the entire adenoviral genome removed, in which case it is preferred that at least the viral inverted terminal repeats

(ITRs) and a packaging signal are left intact (i.e., an adenoviral amplicon). Suitable replication-deficient adenoviral vectors, including multiply replication-deficient adenoviral vectors, are disclosed in U.S. Patents 5,837,511; 5,851,806; 5,994,106; and 6,579,522; U.S. Published Patent Applications 2001/0043922 A1, 2002/0004040 A1, 2002/0031831 A1, and 2002/0110545 A1, and International Patent Applications WO 95/34671, WO 97/12986, and WO 97/21826. Ideally, the pharmaceutical composition is virtually free of replication-competent adenovirus (RCA) contamination (e.g., the pharmaceutical composition comprises less than about 1% of RCA contamination). Most desirably, the pharmaceutical composition is RCA-free. Adenoviral vector compositions and stocks that are RCA-free are described in U.S. Patents 5,944,106 and 6,482,616, U.S. Published Patent Application 2002/0110545 A1, and International Patent Application WO 95/34671. Ideally, the pharmaceutical composition also is free of E1-revertants when the adenoviral vector is E1-deficient in combination with deficiencies in other replication-essential gene functions of another region of the adenoviral genome, as further described in International Patent Application WO 03/040314.

In addition to modification (e.g., deletion, mutation, or replacement) of adenoviral sequences encoding replication-essential gene functions, the adenoviral genome can contain benign or non-lethal modifications, i.e., modifications which do not render the adenovirus replication-deficient, or, desirably, do not adversely affect viral functioning and/or production of viral proteins, even if such modifications are in regions of the adenoviral genome that otherwise contain replication-essential gene functions. Such modifications commonly result from DNA manipulation or serve to facilitate expression vector construction. For example, it can be advantageous to remove or introduce restriction enzyme sites in the adenoviral genome. Such benign mutations often have no detectable adverse effect on viral functioning. For example, the adenoviral vector can comprise a deletion of nucleotides 10,594 and 10,595 (based on the adenoviral serotype 5 genome), which are associated with VA-RNA-1 transcription, but the deletion of which does not prohibit production of VA-RNA-1.

Similarly, the coat protein of a viral vector, preferably an adenoviral vector, can be manipulated to alter the binding specificity or recognition of a virus for a viral receptor on a potential host cell. For adenovirus, such manipulations can include deletion of regions of the fiber, penton, or hexon, insertions of various native or non-native ligands into portions of the coat protein, and the like. Manipulation of the coat protein can broaden the range of cells infected by a viral vector or enable targeting of a viral vector to a specific cell type. For example, in one embodiment, the expression vector is an adenoviral vector comprising a chimeric coat protein (e.g., a fiber, hexon pIX, pIIIa, or penton protein), which differs from the wild-type (i.e., native) coat protein by the introduction of a nonnative amino acid sequence, preferably at or near the carboxyl terminus. Preferably, the nonnative amino acid sequence is inserted into or in place of an internal coat protein sequence. One of ordinary

skill in the art will understand that the nonnative amino acid sequence can be inserted within the internal coat protein sequence or at the end of the internal coat protein sequence. The resultant chimeric viral coat protein is able to direct entry into cells of the viral, i.e., adenoviral, vector comprising the coat protein that is more efficient than entry into cells of a vector that is identical except for comprising a wild-type viral coat protein rather than the chimeric viral coat protein. Preferably, the chimeric virus coat protein binds a novel endogenous binding site present on the cell surface that is not recognized, or is poorly recognized by a vector comprising a wild-type coat protein. One direct result of this increased efficiency of entry is that the virus, preferably, the adenovirus, can bind to and enter numerous cell types which a virus comprising wild-type coat protein typically cannot enter or can enter with only a low efficiency.

In another embodiment of the invention, the expression vector is a viral vector comprising a chimeric virus coat protein not selective for a specific type of eukaryotic cell. The chimeric coat protein differs from the wild-type coat protein by an insertion of a nonnative amino acid sequence into or in place of an internal coat protein sequence. In this embodiment, the chimeric virus coat protein efficiently binds to a broader range of eukaryotic cells than a wild-type virus coat, such as described in International Patent Application WO 97/20051.

Specificity of binding of an adenovirus to a given cell can also be adjusted by use of an adenovirus comprising a short-shafted adenoviral fiber gene, as discussed in U.S. Patent 5,962,311. Use of an adenovirus comprising a short-shafted adenoviral fiber gene reduces the level or efficiency of adenoviral fiber binding to its cell-surface receptor and increases adenoviral penton base binding to its cell-surface receptor, thereby increasing the specificity of binding of the adenovirus to a given cell. Alternatively, use of an adenovirus comprising a short-shafted fiber enables targeting of the adenovirus to a desired cell-surface receptor by the introduction of a nonnative amino acid sequence either into the penton base or the fiber knob.

Of course, the ability of a viral vector to recognize a potential host cell can be modulated without genetic manipulation of the coat protein. For instance, complexing an adenovirus with a bispecific molecule comprising a penton base-binding domain and a domain that selectively binds a particular cell surface binding site enables one of ordinary skill in the art to target the vector to a particular cell type.

Suitable modifications to a viral vector, specifically an adenoviral vector, are described in U.S. Patents 5,543,328; 5,559,099; 5,712,136; 5,731,190; 5,756,086; 5,770,442; 5,846,782; 5,871,727; 5,885,808; 5,922,315; 5,962,311; 5,965,541; 6,057,155; 6,127,525; 6,153,435; 6,329,190; 6,455,314; and 6,465,253; U.S. Published Applications 2001/0047081 A1, 2002/0099024 A1, and 2002/0151027 A1, and International Patent Applications WO 95/02697, WO 95/16772, WO 95/34671, WO 96/07734, WO 96/22378, WO 96/26281, WO 97/20051, WO 98/07865, WO 98/07877, WO 98/40509, WO 98/54346, WO 00/15823, WO 01/58940, and WO 01/92549. Similarly, it will be appreciated that numerous expression

vectors are available commercially. Construction of expression vectors is well understood in the art. Adenoviral vectors can be constructed and/or purified using methods known in the art (e.g., using complementing cell lines, such as the 293 cell line, Per.C6 cell line, or 293-ORF6 cell line) and methods set forth, for example, in U.S. Patents 5,965,358; 5,994,128; 6,033,908; 6,168,941; 6,329,200; 6,383,795; 6,440,728; 6,447,995; and 6,475,757; U.S. Published Application 2002/0034735 A1, and International Patent Applications WO 98/53087, WO 98/56937, WO 99/15686, WO 99/54441, WO 00/12765, WO 01/77304, and WO 02/29388, as well as the other references identified herein. Adeno-associated viral vectors can be constructed and/or purified using the methods set forth, for example, in U.S. Patent 4,797,368 and Laughlin et al., *Gene*, *23*, 65-73 (1983).

The selection of expression vector for use in the inventive method will depend on a variety of factors such as, for example, the host, immunogenicity of the vector, the desired duration of protein production, and the like. As each type of expression vector has distinct properties, a researcher has the freedom to tailor the inventive method to any particular situation. Moreover, more than one type of expression vector can be used to deliver the gene product to the host cell.

The nucleic acid sequence is desirably present as part of an expression cassette, i.e., a particular nucleotide sequence that possesses functions which facilitate subcloning and recovery of a nucleic acid sequence (e.g., one or more restriction sites) or expression of a nucleic acid sequence (e.g., polyadenylation or splice sites). The nucleic acid sequence is preferably located in the E1 region (e.g., replaces the E1 region in whole or in part) of the adenoviral genome. For example, the E1 region can be replaced by a promoter-variable expression cassette comprising the nucleic acid sequence(s). The expression cassette is preferably inserted in a 3'-5' orientation, e.g., oriented such that the direction of transcription of the expression cassette is opposite that of the surrounding adjacent adenoviral genome. In addition to the expression cassette comprising the nucleic acid sequence(s), the adenoviral vector can comprise other expression cassettes containing nucleic acid sequences encoding other products, which cassettes can replace any of the deleted regions of the adenoviral genome. The insertion of an expression cassette into the adenoviral genome (e.g., into the E1 region of the genome) can be facilitated by known methods, for example, by the introduction of a unique restriction site at a given position of the adenoviral genome. As set forth above, preferably all or part of the E3 region of the adenoviral vector also is deleted.

According to the invention, the nucleic acid sequence is operably linked to regulatory sequences necessary for expression, i.e., a promoter. A "promoter" is a DNA sequence that directs the binding of RNA polymerase and thereby promotes RNA synthesis. A nucleic acid sequence is "operably linked" to a promoter when the promoter is capable of directing transcription of that nucleic acid sequence. A promoter can be native or non-native to the nucleic acid sequence to which it is operably linked.

Any promoter (i.e., whether isolated from nature or produced by recombinant DNA or synthetic techniques) can be used in connection with the invention to provide for transcription of the nucleic acid sequence. The promoter preferably is capable of directing transcription in a eukaryotic (desirably mammalian) cell. The functioning of the promoter can be altered by the presence of one or more enhancers and/or silencers present on the vector. "Enhancers" are cis-acting elements of DNA that stimulate or inhibit transcription of adjacent genes. An enhancer that inhibits transcription also is termed a "silencer." Enhancers differ from DNA-binding sites for sequence-specific DNA binding proteins found only in the promoter (which also are termed "promoter elements") in that enhancers can function in either orientation, and over distances of up to several kilobase pairs (kb), even from a position downstream of a transcribed region.

Promoter regions can vary in length and sequence and can further encompass one or more DNA binding sites for sequence-specific DNA binding proteins and/or an enhancer or silencer. Enhancers and/or silencers can similarly be present on a nucleic acid sequence outside of the promoter *per se*. Desirably, a cellular or viral enhancer, such as the cytomegalovirus (CMV) immediate-early enhancer, is positioned in the proximity of the promoter to enhance promoter activity. In addition, splice acceptor and donor sites can be present on a nucleic acid sequence to enhance transcription.

The invention preferentially employs a viral promoter. Suitable viral promoters are known in the art and include, for instance, cytomegalovirus (CMV) promoters, such as the CMV immediate-early promoter, promoters derived from human immunodeficiency virus (HIV), such as the HIV long terminal repeat promoter, Rous sarcoma virus (RSV) promoters, such as the RSV long terminal repeat, mouse mammary tumor virus (MMTV) promoters, HSV promoters, such as the Lap2 promoter or the herpes thymidine kinase promoter (Wagner et al., *Proc. Natl. Acad. Sci.*, *78*, 144-145 (1981)), promoters derived from SV40 or Epstein Barr virus, an adeno-associated viral promoter, such as the p5 promoter, and the like. Preferably, the viral promoter is an adenoviral promoter, such as the Ad2 or Ad5 major late promoter and tripartite leader, a CMV promoter, or an RSV promoter.

Alternatively, the invention employs a cellular promoter, i.e., a promoter that drives expression of a cellular protein. Preferred cellular promoters for use in the invention will depend on the desired expression profile to produce the therapeutic agent(s). In one aspect, the cellular promoter is preferably a constitutive promoter that works in a variety of cell types, such as cells associated with the eye. Suitable constitutive promoters can drive expression of genes encoding transcription factors, housekeeping genes, or structural genes common to eukaryotic cells. For example, the Ying Yang 1 (YY1) transcription factor (also referred to as NMP-1, NF-E1, and UCRBP) is a ubiquitous nuclear transcription factor that is an intrinsic component of the nuclear matrix (Guo et al., *PNAS*, *92*, 10526-10530 (1995)). While the promoters described herein are considered as constitutive promoters, it is understood in the art that constitutive promoters can be upregulated. Promoter analysis shows that the elements

critical for basal transcription reside from –277 to +475 of the YY1 gene relative to the transcription start site from the promoter, and include a TATA and CCAAT box. JEM-1 (also known as HGMW and BLZF-1) also is a ubiquitous nuclear transcription factor identified in normal and tumorous tissues (Tong et al., *Leukemia*, *12*(11), 1733-1740 (1998), and Tong et al., *Genomics*, *69*(3), 380-390 (2000)). JEM-1 is involved in cellular growth control and maturation, and can be upregulated by retinoic acids. Sequences responsible for maximal activity of the JEM-1 promoter has been located at –432 to +101 of the JEM-1 gene relative the transcription start site of the promoter. Unlike the YY1 promoter, the JEM-1 promoter does not comprise a TATA box. The ubiquitin promoter, specifically UbC, is a strong constitutively active promoter functional in several species. The UbC promoter is further characterized in Marinovic et al., *J. Biol. Chem.*, *277*(19), 16673-16681 (2002).

Many of the above-described promoters are constitutive promoters. Instead of being a constitutive promoter, the promoter can be an inducible promoter, i.e., a promoter that is up- and/or down-regulated in response to appropriate signals. For instance, the regulatory sequences can comprise a hypoxia driven promoter, which is active when an ocular disorder is associated with hypoxia. Other examples of suitable inducible promoter systems include, but are not limited to, the IL-8 promoter, the metallothionine inducible promoter system, the bacterial *lacZYA* expression system, the tetracycline expression system, and the T7 polymerase system. Further, promoters that are selectively activated at different developmental stages (e.g., globin genes are differentially transcribed from globin-associated promoters in embryos and adults) can be employed. The promoter sequence that regulates expression of the nucleic acid sequence can contain at least one heterologous regulatory sequence responsive to regulation by an exogenous agent. The regulatory sequences are preferably responsive to exogenous agents such as, but not limited to, drugs, hormones, or other gene products (ideally gene products produced in the eye). For example, the regulatory sequences, e.g., promoter, preferably are responsive to glucocorticoid receptor-hormone complexes, which, in turn, enhance the level of transcription of a therapeutic gene or a therapeutic fragment thereof.

The regulatory sequences can comprise a tissue-specific promoter, i.e., a promoter that is preferentially activated in a given tissue and results in expression of a gene product in the tissue where activated. A tissue-specific promoter suitable for use in the invention can be chosen by the ordinarily skilled artisan based upon the target tissue or cell-type. Preferred tissue-specific promoters for use in the inventive method are specific to ocular tissue, such as a rhodopsin promoter. Examples of rhodopsin promoters include, but are not limited to, a GNAT cone- transducing alpha-subunit gene promoter or an interphotoreceptor retinoid binding protein promoter.

Also preferably, the expression vector comprises a nucleic acid encoding a cis-acting factor, wherein the cis-acting factor modulates the expression of the nucleic acid sequence. Preferably, the cis-acting factor comprises matrix attachment region (MAR) sequences (e.g.,

immunoglobulin heavy chain (Jenunwin et al., *Nature*, *385*(16), 269 (1997)), apolipoprotein B, or locus control region (LCR) sequences, among others. MAR sequences have been characterized as DNA sequences that associate with the nuclear matrix after a combination of nuclease digestion and extraction (Bode et al., *Science*, *255*(5041), 195-197 (1992)). MAR sequences are often associated with enhancer-type regulatory regions and, when integrated into genomic DNA, MAR sequences augment transcriptional activity of adjacent nucleotide sequences. It has been postulated that MAR sequences play a role in controlling the topological state of chromatin structures, thereby facilitating the formation of transcriptionally-active complexes. Similarly, it is believed LCR sequences function to establish and/or maintain domains permissive for transcription. Many LCR sequences give tissue specific expression of associated nucleic acid sequences. Addition of MAR or LCR sequences to the expression vector can further enhance expression of the nucleic acid sequence.

To optimize protein production, preferably the nucleic acid sequence further comprises a polyadenylation site following the coding region of the nucleic acid sequence. Also, preferably all the proper transcription signals (and translation signals, where appropriate) will be correctly arranged such that the nucleic acid sequence will be properly expressed in the cells into which it is introduced. If desired, the nucleic acid sequence also can incorporate splice sites (i.e., splice acceptor and splice donor sites) to facilitate mRNA production. Moreover, if the nucleic acid sequence encodes a protein or peptide, which is a processed or secreted protein or acts intracellularly, preferably the nucleic acid sequence further comprises the appropriate sequences for processing, secretion, intracellular localization, and the like.

In certain embodiments, it may be advantageous to modulate expression of the nucleic acid sequence encoding the gene product. An especially preferred method of modulating expression of a nucleic acid sequence comprises addition of site-specific recombination sites on the expression vector. Contacting an expression vector comprising site-specific recombination sites with a recombinase will either up- or down-regulate transcription of a coding sequence, or simultaneously up-regulate transcription one coding sequence and down-regulate transcription of another, through the recombination event. Use of site-specific recombination to modulate transcription of a nucleic acid sequence is described in, for example, U.S. Patents 5,801,030 and 6,063,627 and International Patent Application WO 97/09439.

The expression vector can comprise a nucleic acid sequence that encodes any gene product. The gene product can be a protein or RNA useful in methods of treatment, diagnostic methods, or useful in ocular-related research. In one embodiment, the gene product is a therapeutic gene product (i.e., a gene product that achieves a beneficial biological effect in a patient). Suitable gene products include, but are not limited to, cytokines, enzymes,

inhibitors of angiogenesis, neurotrophic factors, antibodies, and biologically-active fragments of any of the foregoing.

Preferably, the expression vector of the inventive method comprises a nucleic acid encoding an inhibitor of angiogenesis. The nucleic acid sequence can encode multiple inhibitors of angiogenesis. By "inhibitor of angiogenesis" is meant any factor that prevents or ameliorates neovascularization. One of ordinary skill in the art will understand that complete prevention or amelioration of neovascularization is not required in order to realize a desired biological effect. Therefore, the inventive method contemplates both partial and complete prevention and amelioration of angiogenesis. An inhibitor of angiogenesis includes, for instance, an anti-angiogenic factor, an anti-sense molecule specific for an angiogenic factor, a ribozyme, a small interfering RNA (siRNA, an RNA interfering molecule), a receptor for an angiogenic factor, and an antibody that binds a receptor for an angiogenic factor.

The anti-angiogenic factors contemplated for use in the invention include, for example, pigment epithelium-derived factor, angiostatin, vasculostatin, endostatin, platelet factor 4, heparinase, interferons (e.g., INFα), tissue inhibitor of metalloproteinase 3 (TIMP3), and the like. Such factors prevent the growth of new blood vessels, promote vessel maturation, inhibit permeability of blood vessels, inhibit the migration of endothelial cells, and the like. Various anti-angiogenic factors are described in International Patent Application WO 02/22176. One of ordinary skill in the art will appreciate that any anti-angiogenic factor can be modified or truncated and retain anti-angiogenic activity. As such, active fragments of anti-angiogenic factors (i.e., those fragments having biological activity sufficient to inhibit angiogenesis) are also suitable.

An anti-sense molecule specific for an angiogenic factor should generally be substantially identical to at least a portion, preferably at least about 20 continuous nucleotides, of the nucleic acid encoding the angiogenic factor to be inhibited, but need not be identical. The anti-sense nucleic acid molecule can be designed such that the inhibitory effect applies to other proteins within a family of genes exhibiting homology or substantial homology to the nucleic acid. The introduced anti-sense nucleic acid molecule also need not be full-length relative to either the primary transcription product or fully processed mRNA. Generally, higher homology can be used to compensate for the use of a shorter sequence. Furthermore, the anti-sense molecule need not have the same intron or exon pattern, and homology of non-coding segments will be equally effective. Antisense phosphorothiotac oligodeoxynucleotides (PS-ODNs) is exemplary of an anti-sense molecule specific for an angiogenic factor. Also suitable are other RNA interfering agents, such as siRNA (see, e.g., Chui et al., *Mol. Cell.*, *10*(3), 549-61 (2002)).

Ribozymes can be designed that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered and is, thus, capable of recycling and cleaving other molecules, making it a true enzyme. The inclusion of

ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs. The design and use of target RNA-specific ribozymes is described in Haseloff et al., *Nature, 334*, 585-591 (1988). Preferably, the ribozyme comprises at least about 20 continuous nucleotides complementary to the target sequence on each side of the active site of the ribozyme.

Receptors specific for angiogenic factors inhibit neovascularization by sequestering growth factors away from functional receptors capable of promoting a cellular response. For example, Flt and Flk receptors (e.g., soluble flt (sflt)), as well as VEGF-receptor chimeric proteins, compete with VEGF receptors on vascular endothelial cells to inhibit endothelial cell growth (Aiello, *PNAS, 92*, 10457 (1995)). Also contemplated are growth factor-specific antibodies and fragments thereof (e.g., Fab, $F(ab')_2$, and Fv) that neutralize angiogenic factors or bind receptors for angiogenic factors.

The expression vector can comprise a nucleic acid sequence encoding a vessel maturation factor. Many ocular disorders involve leakage of blood products through vessels, which can cloud vision and induce an immune response within the layers of the eye. Vessel maturation factors reduce the amount of vascular leakage and, therefore, are useful in treating, for example, exudative ocular disorders. Vessel maturation factors include, but are not limited to, angiopoietins (Ang, e.g., Ang-1 and Ang-2), tumor necrosis factor-alpha (TNF-α), midkine (MK), COUP-TFII, hepatic growth factor (HGF), and heparin-binding neurotrophic factor (HBNF, also known as heparin binding growth factor).

The invention also contemplates delivery of a nucleic acid sequence encoding at least one neurotrophic agent (or neurotrophic factor) to ocular cells. Neurotrophic factors are thought to be responsible for the maturation of developing neurons and for maintaining adult neurons. Thus, the method of the invention can be used to inhibit or reverse neural cell degeneration and death not associated with neovascular diseases. Neurotrophic factors are divided into three subclasses: neuropoietic cytokines; neurotrophins; and the fibroblast growth factors. Ciliary neurotrophic factor (CNTF) is exemplary of neuropoietic cytokines. CNTF promotes the survival of ciliary ganglionic neurons and supports certain neurons that are NGF-responsive. Neurotrophins include, for example, brain-derived neurotrophic factor and nerve growth factor, perhaps the best characterized neurotrophic factor. Other neurotrophic factors suitable for being encoded by the nucleic acid sequence of the inventive method include, for example, transforming growth factors, glial cell-line derived neurotrophic factor, neurotrophin 3, neurotrophin 4/5, and interleukin 1-β. Neurotrophic factors associated with angiogenesis, such as aFGF and bFGF, are less preferred. The neurotrophic factor can also be a neuronotrophic factor, e.g., a factor that enhances neuronal survival. It has been postulated that neurotrophic factors can actually reverse degradation of neurons. Such factors, conceivably, are useful in treating the degeneration of neurons associated with vision loss. Neurotrophic factors function in both paracrine and autocrine fashions, making them ideal therapeutic agents. Preferably, the nucleic acid sequence of the

invention encodes both an inhibitor of angiogenesis and a neurotrophic factor. More preferably, the nucleic acid sequence encodes at least one factor comprising both anti-angiogenic and neurotrophic properties. Most preferably, the factor comprising both anti-angiogenic and neurotrophic properties is PEDF.

PEDF, also named early population doubling factor-1 (EPC-1), is a secreted protein having homology to a family of serine protease inhibitors named serpins. PEDF is made predominantly by retinal pigment epithelial cells and is detectable in most tissues and cell types of the body. PEDF has been observed to induce differentiation in retinoblastoma cells and enhance survival of neuronal populations (Chader, *Cell Different.*, *20*, 209-216 (1987)). Factors that enhance neuronal survival under adverse conditions, such as PEDF, are termed "neuronotrophic," as described herein. PEDF further has gliastatic activity, or has the ability to inhibit glial cell growth. As discussed above, PEDF also has anti-angiogenic activity. Anti-angiogenic derivatives of PEDF include SLED proteins, discussed in WO 99/04806. It has also been postulated that PEDF is involved with cell senescence (Pignolo et al., *J. Biol. Chem.*, *268*(12), 8949-8957 (1998)). PEDF for use in the inventive method can be derived from any source, and is further characterized in U.S. Patent 5,840,686 and International Patent Applications WO 93/24529 and WO 99/04806. Desirably, the adenoviral vector comprises the nucleic acid sequence set forth in SEQ ID NO: 1.

The expression vector, e.g., the adenoviral or the adeno-associated viral vector, also can comprise a nucleic acid sequence encoding a protein fragment, such as a therapeutic fragment of at least one inhibitor of angiogenesis or at least one neurotrophic factor. One of ordinary skill in the art will appreciate that any inhibitor of angiogenesis or neurotrophic factor, e.g., PEDF, can be modified or truncated and retain anti-angiogenic or neurotrophic activity. As such, coding sequences for therapeutic fragments (i.e., those fragments having biological activity sufficient to, for example, inhibit angiogenesis or promote neuron survival) also are suitable for incorporation into the expression vector. Also suitable for incorporation into the expression vector are nucleic acid sequences comprising substitutions, deletions, or additions, but which encode a functioning inhibitor of angiogenesis or neurotrophic factor or a therapeutic fragment of any of the foregoing. A functioning inhibitor of angiogenesis or a therapeutic fragment thereof prevents or ameliorates neovascularization. A functioning neurotrophic factor or a therapeutic fragment thereof desirably promotes neuronal cell differentiation, inhibits glial cell proliferation, and/or promotes neuronal cell survival. One of ordinary skill in the art will understand that complete prevention or amelioration of neovascularization is not required in order to realize a therapeutic effect. Likewise, complete induction of neuron survival or differentiation is not required in order to realize a benefit. Therefore, both partial and complete prevention and amelioration of angiogenesis or promotion of neuron survival is appropriate. The ordinarily skilled artisan has the ability to determine whether a modified therapeutic factor or a fragment thereof has neurotrophic and anti-angiogenic therapeutic activity using, for example, neuronal cell

differentiation and survival assays (see, for example, U.S. Patent 5,840,686), the mouse ear model of neovascularization, or the rat hindlimb ischemia model.

Similarly, one of ordinary skill in the art will appreciate that the inhibitor of angiogenesis and/or the neurotrophic factor can be a factor that acts upon a receptor for an anti-angiogenic factor or a receptor for a neurotrophic factor, thereby resulting in the desired biological effect. For instance, the expression vector can comprise a nucleic acid sequence encoding an antibody or peptide agonist that binds and activates the PEDF receptor, which signals a series of intracellular events responsible for the biological activity of PEDF. Likewise, the expression vector can comprise a nucleic acid sequence encoding a peptide that interacts with a PEDF receptor to achieve a biological effect. For example, a dominant positive protein can be constructed which constitutively activates cell-signaling via the PEDF receptor. For a discussion of PEDF receptors, see, for example, Alberdi et al., *J. Biol. Chem.*, *274*(44), 31605 (1999).

The invention also contemplates the use of nucleic acid sequences encoding chimeric or fusion peptides. Through recombinant DNA technology, scientists have been able to generate fusion proteins that contain the combined amino acid sequence of two or more proteins. The ordinarily skilled artisan can fuse the active domains of two or more factors to generate chimeric peptides with desired activity. A fusion protein, such as a fusion protein comprising an anti-angiogenic factor or neurotrophic factor or a therapeutic fragment thereof and for example, a moiety that stabilizes peptide conformation, also can be present in the expression vector. The chimeric peptide can comprise the entire amino acid sequences of two or more peptides or, alternatively, can be constructed to comprise portions of two or more peptides (e.g., 10, 20, 50, 75, 100, 400, 500, or more amino acid residues). Desirably, the chimeric peptide comprises anti-angiogenic and neurotrophic activity, which can be determined using routine methods.

The method of the invention can be part of a treatment regimen involving other therapeutic modalities. It is appropriate, therefore, if the ocular-related disorder, namely ocular neovascularization or age-related macular degeneration, has been treated, is being treated, or will be treated with any of a number of additional ocular therapies, such as drug therapy, panretinal therapy, thermotherapy, radiation therapy, or surgery. The surgery can comprise, for instance, macular translocation, removal of subretinal blood, or removal of subretinal choroidal neovascular membrane. The expression vector is preferably administered intraocularly for the prophylactic or therapeutic treatment of an ocular-related disorder, e.g., age-related macular degeneration or persistent or recurrent ocular neovascularization, which is also treated with drugs, surgery, laser photocoagulation, and/or photodynamic therapies.

The expression vector of the inventive method is delivered to the eye, wherein the expression vector transduces host cells. Delivery to the eye can be achieved by administering the expression vector to any component of the ocular apparatus (e.g., eye globe, layers of the

eye globe, muscles or connective tissue associated with the eye, etc.), such that the gene product is produced and delivered to target ocular cells. Ocular cells include, but are not limited to, cells of neural origin, cells of all layers of the retina, especially retinal pigment epithelial cells, glial cells, pericytes, endothelial cells, iris epithelial cells, corneal cells, ciliary epithelial cells, Mueller cells, astrocytes, muscle cells surrounding and attached to the eye (e.g., cells of the lateral rectus muscle), fibroblasts (e.g., fibroblasts associated with the episclera), orbital fat cells, cells of the sclera and episclera, connective tissue cells, muscle cells, cells of the trabecular meshwork, fibroblasts, and vascular endothelial cells. In that a great deal of retinal damage occurs as a result of edema, thickening of underlying membranes, and build-up of metabolic byproducts, the expression vector can be administered to an area of vascular leakage.

The expression vector desirably is administered in a pharmaceutical composition, which comprises a pharmaceutically acceptable carrier and the expression vector(s). Any suitable pharmaceutically acceptable carrier can be used within the context of the invention, and such carriers are well known in the art. The choice of carrier will be determined, in part, by the particular site to which the composition is to be administered and the particular method used to administer the composition.

Suitable formulations include aqueous and non-aqueous solutions, isotonic sterile solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood or intraocular fluid of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, immediately prior to use. Extemporaneous solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Preferably, the pharmaceutically acceptable carrier is a buffered saline solution. More preferably, the expression vector is administered in a pharmaceutical composition formulated to protect and/or stabilize the expression vector from damage prior to administration. For example, the pharmaceutical composition can be formulated to reduce loss of the expression vector on devices used to prepare, store, or administer the expression vector, such as glassware, syringes, pellets, slow-release devices, pumps, or needles. The pharmaceutical composition can be formulated to decrease the light sensitivity and/or temperature sensitivity of the expression vector. To this end, the pharmaceutical composition preferably comprises a pharmaceutically acceptable liquid carrier, such as, for example, those described above, and a stabilizing agent selected from the group consisting of polysorbate 80, L-arginine, polyvinylpyrrolidone, trehalose, and combinations thereof. In one embodiment, the formulation comprises Tris base (10 mM), NaCl (75 mM), $MgCl \cdot 6H_2O$ (1 mM), polysorbate 80 (0.0025%) and trehalose dehydrate (5%). Use of such a pharmaceutical composition will

extend the shelf life of the vector, facilitate administration, and increase the efficiency of the inventive methods. In this regard, a pharmaceutical composition also can be formulated to enhance transduction efficiency. Suitable compositions are further described in U.S. Patents 6,225,289 and 6,514,943.

In addition, one of ordinary skill in the art will appreciate that the expression vector, e.g., viral vector, of the invention can be present in a composition with other therapeutic or biologically-active agents. For example, therapeutic factors useful in the treatment of a particular indication can be present. For instance, if treating vision loss, hyaluronidase can be added to a composition to, for example, affect the break down of blood and blood proteins in the vitreous of the eye. Factors that control inflammation, such as ibuprofen or steroids, can be part of the composition to reduce swelling and inflammation associated with *in vivo* administration of the viral vector and ocular distress. Inflammation also can be controlled by down-regulating the effects of cytokines involved in the inflammation process (e.g., TNFα). Alternatively, agonists for chemokines which control inflammation (e.g., TGFβ) can be included to reduce the harmful effects of inflammation. Immune system suppressors can be administered in combination with the inventive method to reduce any immune response to the vector itself or associated with an ocular disorder. Anti-angiogenic factors, such as soluble growth factor receptors (sflt), growth factor antagonists (e.g., angiotensin), an anti-growth factor antibody (e.g., Lucentis™), Squalamine (an aminosterol), and the like also can be part of the composition, as well as additional neurotrophic factors. Similarly, vitamins and minerals, anti-oxidants, and micronutrients can be co-administered. Antibiotics, i.e., microbicides and fungicides, can be present to reduce the risk of infection associated with gene transfer procedures and other disorders. Ligands for nuclear receptors such as thyroid hormones, retinoids, specific prostaglandins, estrogen hormone, glucocorticoids or their analogues can be part of the composition. Small molecule agonists for the PEDF receptor also can be included in the formulation. Such small molecule agonists can amplify the therapeutic effect of the inventive method. Suitable drugs for inclusion in the formulation include, but are not limited to, a prostaglandin analogue, a beta-blocker (as commonly used for glaucoma treatment), hyaluronidase (e.g., Vitrase™ available from Allergan), pegaptanib sodium (e.g., Macugen™), tetrahydrozoline hydrochloride (e.g., Visine™), dorzolamide hydrochloride (Cosopt™ and Truspot™), and an alpha-2-adrenergic agonist (e.g., Alphagan™). Alternatively, these compounds can be administered separately to the animal.

One skilled in the art will appreciate that suitable methods, i.e., invasive and noninvasive methods, of administering an expression vector to the eye are available. Although more than one route can be used to administer a particular expression vector to an eye, such as a human eye, a particular route can provide a more immediate and more effective reaction than another route. Accordingly, the described routes of administration are merely exemplary and are in no way limiting.

Any route of administration is appropriate so long as the expression vector transduces a host cell. The expression vector can be appropriately formulated and administered in the form of an injection, eye lotion, ointment, implant and the like. An expression vector can be applied, for example, topically, intracamerally, subconjunctivally, intraocularly, retrobulbarly, periocularly (e.g., subtenon delivery), subretinally, intravitreously, or suprachoroidally for direct administration to the eye. In certain cases, it may be appropriate to administer multiple applications and employ multiple routes, e.g., subretinal and intravitreous, to ensure sufficient exposure of ocular cells to the expression vector. Multiple applications of the expression vector may also be required to achieve the desired effect.

Depending on the particular case, it may be desirable to non-invasively administer the expression vector to a patient. For instance, if multiple surgeries have been performed, the patient displays low tolerance to anesthetic, or if other ocular-related disorders exist, topical administration of the expression vector may be most appropriate. Topical formulations are well known to those of skill in the art. Such formulations are suitable in the context of the invention for application to the skin. The use of patches, corneal shields (see, e.g., U.S. Patent 5,185,152), and ophthalmic solutions (see, e.g., U.S. Patent 5,710,182) and ointments, e.g., eye drops, is also within the skill in the art. The expression vector can also be administered non-invasively using a needleless injection device, such as the Biojector 2000 Needle-Free Injection Management System® available from Bioject, Inc.

The expression vector can be present in or on a device that allows controlled or sustained release of the expression vector, such as an ocular sponge, meshwork, mechanical reservoir, or mechanical implant. Implants (see, e.g., U.S. Patents 4,853,224; 4,997,652; and 5,443,505), devices (see, e.g., U.S. Patents 4,863,457; 5,098,443; 5,554,187; and 5,725,493), such as an implantable device, e.g., a mechanical reservoir, an intraocular device or an extraocular device with an intraocular conduit, or an implant or a device comprised of a polymeric composition are particularly useful for ocular administration of the expression vector. An expression vector also can be administered in the form of sustained-release formulations (see, e.g., U.S. Patent 5,378,475) comprising, for example, gelatin, chondroitin sulfate, a polyphosphoester, such as bis-2-hydroxyethyl-terephthalate (BHET), or a polylactic-glycolic acid.

Alternatively, the expression vector can be administered using invasive procedures, such as, for instance, intravitreal injection or subretinal injection, optionally preceded by a vitrectomy, or periocular (e.g., subtenon) delivery. The expression vector can be injected into different compartments of the eye, e.g., the vitreal cavity or anterior chamber. Pharmaceutically acceptable carriers for injectable compositions are well-known to those of ordinary skill in the art (see *Pharmaceutics and Pharmacy Practice*, J.B. Lippincott Co., Philadelphia, PA, Banker and Chalmers, eds., pages 238-250 (1982), and *ASHP Handbook on Injectable Drugs*, Toissel, 4th ed., pages 622-630 (1986)). Although less preferred, the expression vector can also be administered *in vivo* by particle bombardment, i.e., a gene gun.

Preferably, the expression vector is administered via an ophthalmologic instrument for delivery to a specific region of an eye. Use of a specialized ophthalmologic instrument ensures precise administration of the expression vector while minimizing damage to adjacent ocular tissue. A preferred ophthalmologic instrument is a combination of forceps and subretinal needle or sharp bent cannula.

While intraocular injection is preferred, injectable compositions also can be administered intramuscularly, intravenously, intraarterially, intraperitoneally, parenterally, systemically, or subcutaneously. Expression vectors also can be administered intratracheally, orally, trans-dermally, or intranasally. Preferably, any expression vector administered to a patient using these routes of administration in the context of the invention is specifically targeted to ocular cells. As discussed herein, an expression vector can be modified to alter the binding specificity or recognition of an expression vector for a receptor on a potential host cell. With respect to adenovirus, such manipulations can include deletion of regions of the fiber, penton, or hexon, insertions of various native or non-native ligands into portions of the coat protein, and the like. One of ordinary skill in the art will appreciate that parenteral administration can require large doses or multiple administrations to effectively deliver the expression vector to the appropriate host cells.

One of ordinary skill in the art will also appreciate that dosage and routes of administration can be selected to minimize loss of expression vector due to a host's immune system. For example, for transducing ocular cells *in vivo*, it can be advantageous to administer to a host a null expression vector (i.e., an expression vector not comprising the nucleic acid sequence encoding the gene product) prior to performing the inventive method. Prior administration of null expression vectors can serve to create an immunity (e.g., tolerance) in the host to the expression vector, thereby decreasing the amount of vector cleared by the immune system.

The dose of expression vector administered to an animal, particularly a human, in accordance with the invention should be sufficient to affect the desired response in the animal over a reasonable time frame. One skilled in the art will recognize that dosage will depend upon a variety of factors, including the age, species, the pathology in question, and condition or disease state. Dosage also depends on the gene product, e.g., inhibitor of angiogenesis and/or neurotrophic factor, to be expressed, as well as the amount of ocular tissue to be transduced and/or about to be affected or actually affected by the ocular-related disease. The size of the dose also will be determined by the route, timing, and frequency of administration as well as the existence, nature, and extent of any adverse side effects that might accompany the administration of a particular expression vector and the desired physiological effect. It will be appreciated by one of ordinary skill in the art that various conditions or disease states, in particular, chronic conditions or disease states, may require prolonged treatment involving multiple administrations.

Suitable doses and dosage regimens can be determined by conventional range-finding techniques known to those of ordinary skill in the art. Preferably, about 10^6 viral particles to about 10^{12} viral particles are delivered to the patient. In other words, a pharmaceutical composition can be administered that comprises an expression vector concentration of from about 10^6 particles/ml to about 10^{12} particles/ml (including all integers within the range of about 10^6 particles/ml to about 10^{12} particles/ml), preferably from about 10^{10} particles/ml to about 10^{12} particles/ml, and will typically involve the intraocular administration of from about 0.1 μl to about 100 μl of such a pharmaceutical composition per eye. In some instances, an injection can comprise from about 0.5 mL to about 1 mL of pharmaceutical composition. Ideally, a dose of about 1×10^6, about $1 \times 10^{6.5}$, about 1×10^7, about $1 \times 10^{7.5}$, about 1×10^8, about $1 \times 10^{8.5}$, about 1×10^9, or about $1 \times 10^{9.5}$ particles of adenoviral vector (e.g., about 3×10^7, 3×10^8, or 3×10^9 particles of adenoviral vector) is administered per eye to a patient via intravitreal injection. Alternatively, the adenoviral vector of the inventive method is administered subretinally in a dose of about 1×10^5, about $1 \times 10^{5.5}$, about 1×10^6, about $1 \times 10^{6.5}$, about 1×10^7, about $1 \times 10^{7.5}$, about 1×10^8, or about $1 \times 10^{8.5}$ particles per eye. When administered periocularly, the dose of adenoviral vector administered preferably is about 1×10^7, about $1 \times 10^{7.5}$, about 1×10^8, about $1 \times 10^{8.5}$, about 1×10^9, about $1 \times 10^{9.5}$, about 1×10^{10}, about $1 \times 10^{10.5}$, about 1×10^{11}, about $1 \times 10^{11.5}$, or about 1×10^{12} particles per eye. When the expression vector is a plasmid, preferably about 0.5 ng to about 1000 μg of DNA is administered. More preferably, about 0.1 μg to about 500 μg is administered, even more preferably about 1 μg to about 100 μg of DNA is administered. Most preferably, about 50 μg of DNA is administered per eye. Of course, other routes of administration may require smaller or larger doses to achieve a therapeutic effect. Any necessary variations in dosages and routes of administration can be determined by the ordinarily skilled artisan using routine techniques known in the art.

The expression vector can be administered any time after inducing a stress response in the eye. It is desirable to administer the expression vector after inducing the stress response such that transduction of host cells is enhanced and expression of the nucleic acid sequence (i.e., transcription) is increased as compared to an expression vector administered in the absence of inducing a stress response in the eye. Preferably, the expression vector is administered within 3 months (e.g., within 2 months) of inducing a stress response in the eye (e.g., exposing the eye to photocoagulation or photodynamic therapy). More preferably, the expression vector is administered within 28 days (e.g., within 21 days or within 14 days) of inducing a stress response in the eye. Also preferably, the expression vector is administered to the eye within 7 days of (e.g., 1, 2, 3, 4, 5, 6, or 7 days after) inducing a stress response in the eye. Also preferably, the expression vector is administered within 3 days (e.g., 1, 2, or 3 days) or within 1 day of inducing the stress response in the eye.

In some embodiments, it is advantageous to administer two or more (i.e., multiple) doses of the expression vector comprising a nucleic acid sequence encoding a gene product to

achieve the desired biological response. The inventive method provides for multiple applications of the expression vector. For example, at least two applications of an expression vector comprising an exogenous nucleic acid encoding a gene product, e.g., a nucleic acid sequence encoding at least one inhibitor of angiogenesis and/or at least one neurotrophic agent, can be administered to the same eye. Preferably, the multiple doses are administered while retaining gene expression above background levels. Also preferably, two applications or more of the expression vector is administered within about 30 days or more. More preferably, two or more applications are administered to the same eye within about 90 days or more. However, three, four, five, six, or more doses can be administered in any time frame (e.g., 2, 7, 10, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 85 or more days between doses) so long as gene expression occurs (and preferably the ocular disorder is inhibited or ameliorated).

As discussed herein, the expression vector of the inventive method comprises a nucleic acid sequence that encodes a gene product (e.g., at least one inhibitor of angiogenesis and/or at least one neurotrophic factor). The nucleic acid sequence can encode multiple, i.e., two, three, or more, gene products, or comprise additional transgenes. Desirably, expression of one or more additional gene products is beneficial, e.g., prophylactically or therapeutically beneficial, to the ocular cell or eye. If the gene product confers a prophylactic or therapeutic benefit to the cell, the transgene can exert its effect at the level of RNA or protein. For example, a peptide other than an inhibitor of angiogenesis or neurotrophic factor that can be employed in the treatment or study of a disorder, e.g., an ocular-related disorder, can be encoded by the expression vector. Alternatively, a nucleic acid sequence encoding an antisense molecule, a ribozyme, siRNA, a protein that affects splicing or 3' processing (e.g., polyadenylation), or a protein that affects the level of expression of another gene within the cell (i.e., where gene expression is broadly considered to include all steps from initiation of transcription through production of a process protein), such as by mediating an altered rate of mRNA accumulation or transport or an alteration in post-transcriptional regulation, can be included in the expression vector. In a preferred embodiment, the nucleic acid sequence encodes PEDF and ciliary neurotrophic factor (CNTF) or sflt. Multiple inhibitors of angiogenesis and/or multiple neurotrophic factors can be operably linked to different promoters. Multiple gene products can be encoded by multiple expression vectors, which are administered to the eye and produced within a target cell. The transgene can encode a chimeric peptide for combination treatment of an ocular-related disorder.

A nucleotide sequence encoding an immunosuppressor also can be incorporated into the expression vector to reduce any inappropriate immune response within the eye as a result of an ocular-related disorder or the administration of the expression vector.

A transgene encoding a marker protein, such as green fluorescent protein or luciferase, can be incorporated into the expression vector. Such marker proteins are useful in vector construction and determining vector migration. Marker proteins also can be used to

determine points of injection or treated ocular tissues in order to efficiently space injections of the expression vector to provide a widespread area of treatment, if desired.

The inventive method also can involve the co-administration of other pharmaceutically active compounds. By "co-administration" is meant administration before, concurrently with, e.g., in combination with the expression vector in the same formulation or in separate formulations, or after administration of the expression vector as described above. Any of the exogenous materials, drugs, proteins, and the like described herein can be co-administered with the expression vector as adjuvant therapy. For example, factors that control inflammation, such as ibuprofen or steroids, can be co-administered to reduce swelling and inflammation associated with intraocular administration of the expression vector. Immunosuppressive agents can be co-administered to reduce inappropriate immune responses related to an ocular disorder or the practice of the inventive method. Anti-angiogenic factors, such as soluble growth factor receptors, growth factor antagonists, i.e., angiotensin, and the like can also be co-administered, as well as neurotrophic factors. In addition, the expression vector of the inventive method can be administered with anti-proliferative agents such as siRNA, aptamers, or antibodies which sequester or inactivate angiogenic factors such as, for example, VEGF. Similarly, vitamins and minerals, anti-oxidants, and micronutrients can be co-administered. Antibiotics, i.e., microbicides and fungicides, can be co-administered to reduce the risk of infection associated with ocular procedures and some ocular-related disorders. Other therapeutics for ocular disorders can be administered in conjunction with the inventive method. For example, Visudyne® (Novartis), Macugen™ (Pfizer), Retaane™ (Alcon), Lucentis™ (Genentech/Novartis), Squalamine (Genaera), Cosopt, and Alphagan can be formulated with the expression vector or can be administered separately before, during, or after administration of the expression vector to the animal.

EXAMPLES

The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLE 1

This example demonstrates the ability of an expression vector comprising a nucleic acid sequence encoding PEDF to inhibit choroidal neovascularization (CNV).

Replication-deficient (E1-/E3-deficient) adenoviral vectors (AdPEDF.10) comprising the coding sequence for PEDF operably linked to the CMV immediate early promoter were constructed using standard techniques. A null version of the vector (AdNull.10), which did not comprise the PEDF coding sequence, was also constructed.

Adult C57BL/6 mice were injected intravitreously with AdNull.10 or AdPEDF.10 using a Harvard pump microinjection apparatus and pulled glass micropipettes. Each eye was

injected intravitreously with 1 μl of vehicle containing 10^9 particles of virus. Alternatively, each eye was injected subretinally with 10^8 particles of virus suspended in 1 μl of vehicle. Five days post-injection, mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight). Topicamide (1%) was utilized to dilate the pupils prior to rupture of Bruch's membrane by diode laser photocoagulation. Rupture of Bruch's membrane is known to induce neovascularization of the choroid.

Fourteen days following laser-induced rupture of Bruch's membrane, choroidal flat mounts (described in Edelman et al., *Invest. Ophthalmol. Vis. Sci.*, *41*, S834 (2000)) were prepared to observe the degree of neovascularization of the choroidal membrane. Briefly, eyes were removed from the subjects and fixed in phosphate-buffered formalin. The cornea, lens, and retina were removed from the eyecup, and the eyecup was flat-mounted. Flat mounts were then examined by fluorescence microscopy and images were digitized using a 3 color CCD video camera (IK-TU40A, Toshiba, Tokyo, Japan) for computer image analysis.

Large areas of neovascularization were observed in uninjected eyes and eyes receiving AdNull.10. Eyes injected with AdPEDF.10 subretinally or intravitreously showed smaller regions of neovascularization compared to the controls using computerized image analysis.

The above results illustrate the ability PEDF, delivered to the eye using an adenoviral vector, to inhibit ocular neovascularization, namely choroidal neovascularization (CNV), in a clinically animal relevant model.

EXAMPLE 2

This example demonstrates the ability of an expression vector comprising a nucleic acid sequence encoding PEDF to inhibit ischemia-induced retinal neovascularization.

Replication-deficient adenoviral vectors comprising the coding sequence for PEDF operably linked to the CMV immediate early promoter were constructed using standard techniques. E1-/E3-/E4-deficient vectors encoding PEDF (AdPEDF.11) and a null version of the vector (AdNull.11), which did not comprise the PEDF coding sequence, were constructed.

Ischemic retinopathy was produced in adult C57BL/6 mice as previously described (see, for example, Smith et al., *Invest. Ophthalmol. Vis. Sci.*, *35*, 101 (1994)). Briefly, seven day old mice (P7) were exposed to an atmosphere of 75 +/- 3% oxygen for five days. At P10, mice were injected intravitreously with 10^9 particles of AdPEDF.11 or AdNull.11, returned to oxygen for two days, then returned to room atmosphere. At P17, the mice were sacrificed and eyes were rapidly removed and frozen in optimum cutting temperature embedding compound (OCT; Miles Diagnostics, Elkhart, IN).

To detect neovascularization, the eyes were sectioned and histochemically stained with biotinylated griffonia simplicifolia lectin B4 (GSA, Vector Laboratories, Burlingame, CA). Slides were then incubated in methanol/H_2O_2 for 10 minutes at 4 °C, washed with 0.05

M Tris-buffered saline, pH 7.6 (TBS), and incubated for 30 minutes in 10% normal porcine serum. The slides were then incubated for two hours with biotinylated GSA, rinsed with TBS, and incubated with avidin-coupled alkaline phosphatase (Vector Laboratories) for 45 minutes. After a 10 minute wash with TBS, the slides were incubated with Histomark Red. GSA-stained, 10 μm serial sections were examined using an Axioskop microscope. Images were digitized using a 3 color CCD video camera (IK-TU40A, Toshiba, Tokyo, Japan) for computer image analysis.

Extensive retinal neovascularization was detected in eyes not injected with any virus. Eyes injected with AdNull.11 showed less neovascularization than uninjected eyes, but significantly more neovascularization of the retina than eyes injected with AdPEDF.11. Eyes injected with AdPEDF.11 comprised the least amount of neovascularization.

This example clearly demonstrates the ability of adenoviral vector-mediated delivery of PEDF to inhibit an ocular-related disorder, namely ischemia-induced retinal neovascularization, in a clinically relevant animal model.

EXAMPLE 3

This example demonstrates the ability of the inventive method to deliver a gene product to the eye.

Forty albino Lewis rats were used in the study, a portion of which were administered photocoagulation therapy or photodynamic therapy. Half of the posterior fundus was laser treated, and the remaining hemisphere was untreated as an internal control. The remaining rats were not pre-treated with laser therapy and served as negative controls. An adenoviral vector comprising an adenoviral genome deficient in one or more essential gene functions of the E1, E3, and E4 regions of the adenoviral genome and comprising the β-galactosidase gene (AdZ.11) was injected intravitreously using Hamilton syringes. The β-galactosidase gene under the control of the CMV immediate early promoter replaced the E1 region of the adenoviral genome while the E4 region was replaced with a spacer sequence that is not transcribed.

Rats received an intravitreous injection of 3×10^9 particles of AdZ.11 per eye at 1, 3, 7, and 28 days after laser treatment. Five days after adenovector administration, the eyes were enucleated and examined histochemically. LacZ expression was greatest when the adenoviral vector was administered three days following laser treatment. However, gene expression also was enhanced as compared to controls (eyes wherein AdZ.11 was administered without laser pre-treatment) when AdZ.11 was administered 1, 7, and 28 days after laser treatment. Retina pre-treated with photodynamic therapy showed less frequent enhanced gene expression than photocoagulation-treated retina.

To examine the persistence of nucleic acid expression, rats were administered 3×10^9 particles of AdZ.11 per eye three days following laser treatment. Eyes were enucleated and examined histochemically at 5, 14, 28, and 90 days after adenovector administration.

Expression of the nucleic acid sequence encoding the β-galactosidase gene product was enhanced at all timepoints examined and was sustained at 90 days after administration of AdZ.11 (the longest duration examined) in photocoagulation-treated animals.

A study of various doses in the context of the inventive method was performed. Rats received intravitreous injection of 3×10^7 particles, 3×10^8 particles, or 3×10^9 particles of AdZ.11 per eye three days following laser photocoagulation. Eyes were enucleated and examined histochemically five days after adenovector administration. Transduction and lacZ expression was enhanced at all dose levels, including the dose of 3×10^7 viral particles, the lowest dose examined.

In summary, enhanced transduction and gene expression were observed in eyes exposed to photodynamic or photocoagulation therapy prior to administration of an adenoviral vector comprising a nucleic acid sequence encoding a gene product. Expression of the nucleic acid sequence was enhanced when the expression vector was administered 1, 3, 7, and 28 days after laser pre-treatment. Enhanced gene expression was observed at all timepoints examined after viral delivery and was sustained to 90 days, the longest timepoint examined. The data provided by this example demonstrates the ability of the inventive method to deliver a gene product to the eye, and effect enhanced and prolonged gene expression in the eye.

EXAMPLE 4

This example demonstrates enhanced transgene expression in the eye as a result of the inventive method.

Female Lewis rats were obtained at 4 to 8 weeks of age, and were administered photocoagulation therapy or photodynamic therapy (PDT). Specifically, photocoagulation therapy involved delivery of a thermal diode laser PC (532 nm wavelength, 200 μm spot size, 0.5 second duration, 300 mW) using the slitlamp delivery system (SL130, Zeiss, Germany) and a hand-held cover slide as a contact lens. Fifty burns were confined to the left half of the posterior fundus in each eye. PDT was performed using the hydrophilic photosensitizer mono-L-aspartyl chlorin e6 (NPe6, LS11, Light Science, Seattle, WA) (see, e.g., Mori et al., *Ophthalmology, 106*, 1384-1391 (1999), Peyman et al., *Ophthalmology, 107*, 29-35 (2000), and Mori et al., *Retina, 21*, 499-508 (2001)). A solution of 10 mg/kg body weight LS11 was administered to each rat via the tail vein. Irradiation of the diode laser (664 nm wavelength, 100 μm spot size, 10 second duration, 4.5 mW) was started within five minutes after intravenous injection. Five laser spots were placed in the left half of the posterior fundus in each eye, using a 664 nm diode laser delivery system. Half of the posterior fundus was laser treated, and the remaining hemisphere was untreated as an internal control. Rats not pre-treated with laser therapy served as negative controls.

One group of rats was administered a dose of 3×10^9 particle units (pu) of AdZ.11 (Example 3) per eye on day 1, 3, 7, and 28 after laser treatment. The adenoviral vector was

injected intravitreously using Hamilton syringes. Five days after adenoviral vector injection, eyes were enucleated and examined histochemically for LacZ staining. Histochemical analysis consisted of fixing eyes in 2.5% glutaraldehyde in phosphate buffer solution (PBS) for 60 minutes and rinsing five times for 10 minutes in PBS. The anterior segments were removed and the remaining posterior segments were incubated overnight in 1 mg/mL 5-bromo-4-chloro-3-indolyl galactopyranoside (X-gal, Sigma, St. Louis, MO) in a solution containing 5mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)6\text{-}3H_2O$, and 1 mM $MgCl_2$ in PBS. Eyes were post-fixed for 15 minutes and then rinsed with PBS. For quantitative analysis of X-gal staining in the posterior segment of eyes, a dissecting microscope and its camera system (MZ 8 and MPS 30, Leica, Wetzlar, Germany) were used. Images were captured, digitized and analyzed by image-analysis software (Win ROOF, Mitani Corporation, Fukui, Japan) to delineate X-gal stained areas. Area ratios (X-gal stained area to total retinal area) were calculated for each eye. Statistical analysis comparing the area ratio between treated left hemisphere and the untreated right hemisphere was performed using Wilcoxon signed-ranks test. A p-value of less than 0.05 was prospectively assigned as the level at which a finding would be considered statistically significant.

There was a significant difference in the ratio of LacZ staining between the photocoagulation-treated left fundus and untreated right fundus hemispheres on days 1, 3, and 7. There also was a significant difference in the ratio of LacZ staining between the PDT-treated left fundus and untreated right fundus hemispheres on day 3. Retina pre-treated with photodynamic therapy showed less frequent enhanced gene expression than photocoagulation-treated retina.

To examine the persistence of nucleic acid expression, rats were administered 3×10^9 particles of AdZ.11 per eye three days following laser treatment. Eyes were enucleated and examined histochemically at 5, 14, 28, 90, 135, and 180 days after adenoviral vector administration. Expression of the nucleic acid sequence encoding the β-galactosidase gene product was enhanced in the photocoagulation-treated left fundus hemisphere at 5, 14, 90, and 135 days after adenoviral vector administration ($p<0.05$). In contrast, PDT-treated eyes demonstrated enhanced β-galactosidase gene expression only 5 days after injection ($p<0.05$).

To examine the effect of adenoviral vector dose on nucleic acid expression, rats were intravitreously administered 3×10^5 particles, 3×10^6 particles, 3×10^7 particles, 3×10^8 particles, or 3×10^9 particles of AdZ.11 per eye three days following laser photocoagulation. Eyes were enucleated and examined histochemically five days after AdZ.11 administration. Vector transduction and lacZ expression were enhanced at all dose levels ($p<0.05$).

This example demonstrates the ability of the inventive method to deliver a gene product to the eye, and effect enhanced and prolonged gene expression in the eye.

EXAMPLE 5

This example demonstrates the expression of adenovirus cell surface receptors in retinal and choroidal tissues following photocoagulation therapy (PC) or photodynamic therapy (PDT).

Five C57/BL6 mice received fundus PC with a 532 nm diode laser. Untreated mice served as controls. Twenty-four hours after treatment, eyes were enucleated and total RNA was extracted from laser photocoagulated retina and choroid. The samples were processed for quantitative real-time PCR (qRT-PCR) using a sequence detection system (ABI prism 7700, Perkin-Elmer, Foster City, CA) to quantify mRNA expression of coxsackie adenovirus receptor (CAR), the integrins αV, β3, and β5 which mediate cell surface interaction and internalization of adenovirus (see, e.g., Wickham et al., *Cell*, *73*, 309-319 (1993), and Bergelson et al., *Science*, *275*, 1320-1323 (1997)).

Specifically, total RNA was isolated from samples composed of the retina and choroids, including retinal pigment epithelium. The samples were homogenized in TRIzol reagents (LifeTechnologies, Greand Island, NY) and treated with RNase-free DNase (DNase I, Gibco, Paisley, UK) to remove genomic DNA contamination. First-strand cDNA was synthesized by reverse transcription of total RNA using reverse transcriptase (Superscript II, Invitrogen, Carlsbad, CA) with random hexamers as primers in a total reaction volume of 20 μL. Amplification of the control gene ARP (acidic ribosomal phosphoprotein P0) was used for normalization (see, e.g., Simpdon et al., *Mol. Vis.*, *6*, 178-183 (2000), and Hackam et al., *Mol. Vis.*, *10*, 637-649 (2004)). Nucleic acid sequences encoding CAR, integrins αV, β3, and β5, and ARP were amplified using commercially available primers and probe sets (assay IDs: Mm00438361_m1 for CAR, Mm00434506_m1 for integrin αV, Mm00443980_m1 for integrin β3, Mm00439825_m1 for integrin β5, and Mm00725448_s1 for ARP, Applied Biosystems, Foster City, CA). The expression levels of CAR and integrins were assigned arbitarary units (relative to baseline samples) using the comparative Ct methods (see Hackam et al., *supra*, and Davidson et al., *Nature*, *425*, 300-306 (2003)). All qRT-PCR experiments were performed following the guidelines supplied by Applied Biosystems. Statistical analysis was performed using Student's unpaired t-test. A p-value of less than 0.05 was prospectively assigned as the level at which a finding would be considered statistically significant.

No significant difference in mRNA expression was measurable for CAR or any of the integrin genes when photocoagulated retina was compared to untreated retina. The mean mRNA expression for CAR and integrin ß5 in photocoagulated choroid was approximately two-fold higher than untreated choroid, but this difference was not statistically significant ($p>0.05$).

This example demonstrates that adenovirus cell surface receptors are not overexpressed in retinal or choroidal tissues.

All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIMS

1. A method of delivering a gene product to an eye, wherein the method comprises (a) inducing a stress response in the eye, and (b) subsequently administering to the eye an expression vector comprising a nucleic acid sequence encoding a gene product, wherein the expression vector transduces a host cell and the nucleic acid sequence is expressed to produce the gene product.

2. A method of prophylactically or therapeutically treating an animal for an ocular-related disorder, wherein the method comprises (a) inducing a stress response in an eye, and (b) subsequently administering to the eye an expression vector comprising a nucleic acid sequence encoding an inhibitor of angiogenesis and/or a neurotrophic agent such that the expression vector transduces a host cell and the nucleic acid sequence is expressed to produce the inhibitor of angiogenesis and/or neurotrophic agent to treat prophylactically or therapeutically the ocular-related disorder.

3. The method of claim 1 or 2, wherein inducing a stress response in the eye comprises applying photodynamic therapy to the eye.

4. The method of claim 1 or 2, wherein inducing a stress response in the eye comprises applying photocoagulation therapy to the eye.

5. The method of any of claims 1-4, wherein the expression vector is administered to the eye within 28 days after inducing a stress response in the eye.

6. The method of any of claims 1-5, wherein the expression vector is administered to the eye within 7 days after inducing a stress response in the eye.

7. The method of any of claims 1-6, wherein the expression vector is administered within 3 days after inducing a stress response in the eye.

8. The method of any of claims 1-6, wherein the expression vector is administered within 1 day after inducing a stress response in the eye.

9. The method of any of claims 1-8, wherein level of transduction of host cells by the expression vector is enhanced as compared to the level of transduction of host cells by the expression vector in the absence of inducing a stress response in the eye.

10. The method of any of claims 1-9, wherein expression of the nucleic acid sequence is enhanced as compared to expression of the nucleic acid sequence in the absence of inducing a stress response in the eye.

11. The method of any of claims 1-10, wherein expression of the nucleic acid sequence is enhanced for five days post-administration of the expression vector as compared to expression of the nucleic acid sequence in the absence of inducing a stress response in the eye.

12. The method of any of claims 1, 2, or 4-11, wherein expression of the nucleic acid sequence is enhanced for 14 days post-administration of the expression vector as compared to expression of the nucleic acid sequence in the absence of inducing a stress response in the eye.

13. The method of any of claims 1, 2, or 4-12, wherein expression of the nucleic acid sequence is enhanced for 28 days post-administration of the expression vector as compared to expression of the nucleic acid sequence in the absence of inducing a stress response in the eye.

14. The method of any of claims 1, 2, or 4-13, wherein expression of the nucleic acid sequence is enhanced for 90 days post-administration of the expression vector as compared to expression of the nucleic acid sequence in the absence of inducing a stress response in the eye.

15. The method of any of claims 1-14, wherein the expression vector is a viral vector.

16. The method of any of claims 1-15, wherein the expression vector is an adenoviral vector.

17. The method of claim 16, wherein the adenoviral vector is replication-deficient.

18. The method of claim 17, wherein the adenoviral vector is deficient in at least one replication-essential gene function of the E1 region of the adenoviral genome of the adenoviral vector.

19. The method of claim 17 or claim 18, wherein the adenoviral vector is deficient in at least one replication-essential gene function of the E4 region of the adenoviral genome of the adenoviral vector.

20. The method of any of claims 1 or 3-19, wherein the gene product is a protein, and the protein is a cytokine, an inhibitor of angiogenesis, a neurotrophic agent, an enzyme, a vessel maturation factor, or an antibody.

21. The method of claim 20, wherein the gene product is an inhibitor of angiogenesis, and the inhibitor of angiogenesis is soluble flt or pigment epithelium-derived factor (PEDF).

22. The method of any of claims 1-21, wherein the expression vector is administered topically, subconjunctivally, retrobulbarly, periocularly, intravitreously, subretinally, suprachoroidally, or intraocularly.

23. The method of any of claims 2-22, wherein the ocular-related disorder is ocular neovascularization.

24. The method of any of claims 2-22, wherein the ocular-related disorder is age-related macular degeneration.

25. The method of any of claims 2-22, wherein the ocular-related disorder is retinal tumors.

26. The method of any of claims 2-22, wherein the ocular-related disorder is diabetic retinopathy.

27. The method of any of claims 2-22, wherein the ocular-related disorder is macular edema.

28. The method of any of claims 2-22, wherein the ocular-related disorder is glaucoma.

29. The method of any of claims 2-22, wherein the ocular-related disorder is a retinal degenerative disease.

30. The method of any of claims 1-29, wherein the adenovirus is a human adenovirus.

SEQUENCE LISTING

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MORI, KEISUKE

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/JP2005/008401

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 2003, ANZAI K ET AL: "BOTH LASER PHOTOCOAGULATION AND PHOTODYNAMIC THERAPY ENHANCE GENE TRANSFER IN THE RAT RETINA MEDIATED BY ADENOVIRAL VECTOR" XP002339508 Database accession no. PREV200300511847 the whole document & ARVO ANNUAL MEETING ABSTRACT SEARCH AND PROGRAM PLANNER, vol. 2003, 2003, page Abstract No. 439, ANNUAL MEETING OF THE ASSOCIATION FOR RESEARCH IN VISION AND OPHTHALMOLOGY; FORT LAUDERDALE, FL, USA; MAY 04-08, 2003 -/--	1-30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 August 2005	18/08/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Morawetz, R

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/JP2005/008401

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TAKAHASHI K ET AL: "Intraocular expression of endostatin reduces VEGF-induced retinal vascular permeability, neovascularization, and retinal detachment" FASEB JOURNAL (FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL BIOLOGY), BETHESDA, US, vol. 17, no. 8, May 2003 (2003-05), pages 896-898, XP002267463 ISSN: 0892-6638 the whole document	1-30
Y	CAMPOCHIARO P A: "GENE THERAPY FOR RETINAL AND CHOROIDAL DISEASES" EXPERT OPINION ON BIOLOGICAL THERAPY, ASHLEY, LONDON, GB, vol. 2, no. 5, June 2002 (2002-06), pages 537-544, XP009025756 ISSN: 1471-2598 the whole document	1-30
Y	US 6 174 861 B1 (O'REILLY MICHAEL S ET AL) 16 January 2001 (2001-01-16) the whole document	1-30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2005/008401

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-30 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/JP2005/008401

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6174861 B1	16-01-2001	US 2002155987 A1	24-10-2002
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